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Abstract: The prognosis of patients afflicted by glioblastoma remains poor. Biomarkers for the disease would be desirable in order to allow for an early detection of tumor progression or to indicate rapidly growing tumor subtypes requiring more intensive therapy. In this study, we investigated whether a blood-derived specific miRNA fingerprint can be defined in patients with glioblastoma. To this end, miRNA profiles from the blood of 20 patients with glioblastoma and 20 age- and sex-matched healthy controls were compared. Of 1158 tested miRNAs, 52 were significantly deregulated, as assessed by unadjusted Student's t-test at an alpha level of 0.05. Of these, two candidates, miR-128 (up-regulated) and miR-342-3p (down-regulated), remained significant after correcting for multiple testing by Benjamini-Hochberg adjustment with a p-value of 0.025. The altered expression of these two biomarkers was confirmed in a second cohort of glioblastoma patients and healthy controls by real-time PCR and validated for patients who had received neither radio- nor chemotherapy and for patients who had their glioblastomas resected more than 6 months ago. Moreover, using machine learning, a comprehensive miRNA signature was obtained that allowed for the discrimination between blood samples of glioblastoma patients and healthy controls with an accuracy of 81% [95% confidence interval (CI) 78-84%], specificity of 79% (95% CI 75-83%) and sensitivity of 83% (95% CI 71-85%). In summary, our proof-of-concept study demonstrates that blood-derived glioblastoma-associated characteristic miRNA fingerprints may be suitable biomarkers and warrant further exploration.

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A specific miRNA signature in the peripheral blood of glioblastoma patients

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ABSTRACT

The prognosis of patients afflicted by glioblastoma remains poor. Biomarkers for the disease would be desirable in order to allow for an early detection of tumor progression or to indicate rapidly growing tumor subtypes requiring more intensive therapy. Here, we investigated whether a blood-derived specific miRNA fingerprint can be defined in patients with glioblastoma. To this end, miRNA profiles from the blood of 20 patients with glioblastoma and 20 age- and sex-matched healthy controls were compared. Of 1158 tested miRNAs, 52 were significantly deregulated, as assessed by unadjusted Student's t-test at an alpha level of 0.05. Of these, 2 candidates, miR-128 (up-regulated) and miR-342-3p (down-regulated), remained significant after correcting for multiple testing by Benjamini Hochberg adjustment with a p-value of 0.025. The altered expression of these two biomarkers was confirmed in a second cohort of glioblastoma patients and healthy controls by real-time PCR and validated for patients who had received neither radio- nor chemotherapy and for patients who had their glioblastomas resected more than 6 months ago. Moreover, using machine learning, a comprehensive miRNA signature was obtained that allowed for the discrimination between blood samples of glioblastoma patients and healthy controls with an accuracy of 81% (95% confidence interval (CI) 78%-84%), specificity of 79% (95% CI 75%-83%) and sensitivity of 83% (95% CI 71%-85%). In summary, our proof-of-concept study demonstrates that blood-derived glioblastoma-associated characteristic miRNA fingerprints may be suitable biomarkers and warrant further exploration.

Running title: Glioblastoma-associated miRNA profile in peripheral blood

Key words: glioblastoma, microRNA, biomarker

INTRODUCTION

Glioblastoma remains a major clinical challenge with median survival times around 5 months in population-based studies (Ohgaki and Kleihues 2005) and approximately 15 months within clinical trials (Stupp et al. 2005). The dismal prognosis is due to the infiltrative growth of the tumor and its resistance to conventional therapies leading to rapid tumor recurrence or progression. Monitoring of glioma growth during or after completed therapy always requires expensive procedures such as magnetic resonance imaging (MRI). In this regard, there is a major interest in developing biomarker strategies that allow for a less extensive monitoring of the disease in shorter time intervals. Further, patients at high risk for surgery-associated mortality or small tumors in eloquent areas of the brain could benefit from the discovery of biomarkers for the confirmation of glioblastoma in order to avoid biopsy.

Biomarkers are helpful tools for the diagnosis and monitoring of patients suffering from different tumors. The clinically available molecules include - among others - *prostate specific antigen* for prostate cancer (Reed and Parekh 2010), *carcinoembryonic antigen* for gastrointestinal tumors (Denlinger and Cohen 2007), *CA19-9* for pancreatic neoplasms (Boeck et al. 2006) and *alpha fetoprotein* for hepatocellular carcinoma (Chan et al. 2009). As a common feature, these proteins can easily be determined in the blood. However, no such protein biomarker is available for patients suffering from glioblastoma (Weller et al. 2010). Therefore, we assessed the suitability of blood-derived microRNAs (miRNAs) as potential diagnostic tools. miRNAs are short (17-24 nucleotides) non-coding RNAs which act as post-transcriptional regulators of gene expression (Guarnieri and DiLeone 2008). They are involved in the regulation of physiological processes and differentially regulated under various pathological conditions (Calin and Croce 2006; Zhang et al. 2007). Deregulated miRNAs have been described in glioma cells and glioma tissue compared to normal brain and

several reports indicate that miRNAs contribute to the malignant phenotype of glioma cells (Malzkorn et al. 2010; Rao et al. 2010).

More recently it became clear that disease-associated miRNAs are also detectable in blood, both in the cellular fraction and in serum (Chen et al. 2008). miRNAs may therefore represent useful and easy accessible molecules that can be exploited for diagnostic purposes (Chen et al. 2008; Gilad et al. 2008). miRNA samples from blood can be analyzed by microarrays, real-time PCR or next generation sequencing (NGS). Microarrays and PCR require only low amounts of RNA and are cost-effective compared to NGS. Further, PCR is in routine clinical use for different diagnostic approaches.

Complex miRNA expression patterns, which usually contain more diagnostic information than single biomarkers, have already been used to define specific miRNA fingerprints in the blood associated with different tumor entities and non-neoplastic diseases (Keller et al. 2009b; Häusler et al. 2010; Hu et al. 2010). Accordingly, we explored blood-derived miRNA profiles from glioblastoma patients and healthy controls for their diagnostic potential using contemporary and comprehensive miRNA arrays. The results of this proof-of-principle study may help to establish novel approaches for tumor growth surveillance and prediction of responses to therapy in glioblastoma patients.

MATERIALS AND METHODS

Samples

The study was approved by the ethics committee of the canton of Zurich, Switzerland (E-72/2009). All donors gave written informed consent before blood sampling. Twenty patients with a histopathological diagnosis of glioblastoma were included. The median age was 55 years, ranging from 44-66 years. A detailed overview on the patients' characteristics is given in Table 1. The control population consisted of 20 age- and sex-matched controls without acute or chronic diseases (median age: 58 years, age range 27-75 years). For validation, a set of 10 additional samples from glioblastoma patients (Table 2) and 10 healthy volunteers was collected. The median age was 59 for glioblastoma patients and 56 for healthy control donors.

miRNA extraction and microarray screening

PAXgene Blood RNA tubes (BD Biosciences, Basel, Switzerland) were used for blood sampling. Total RNA was extracted from the cellular fractions using the miRNeasy kit (Qiagen GmbH, Hombrechtikon, Switzerland). To this end, blood cells were pelleted by centrifugation and resuspended in 10 ml RNase-free water. Total RNA including the miRNA was isolated and then purified according to the manufacturer's protocol. RNA was eluted with 40 µl RNase-free water. Subsequently, the RNA samples were frozen at -80°, shipped on dry ice and subjected to analysis by the Geniom "miRNA *homo sapiens*" Biochip on a Geniom real-time analyzer (GRTA, febit gmbh, Heidelberg, Germany). Each micro-fluidic device contained five replicates of 1158 known human mature miRNAs as annotated in the Sanger miRBase 14.0 (Griffiths-Jones et al. 2005; Griffiths-Jones et al. 2008). Before hybridization, no external labelling or amplification was carried out. All samples were processed fully automated on the GRTA following quality control. For labelling, a microfluidic-based primer extension assay (MPEA) was carried out (Vorwerk et al. 2008). In more detail, 300 ng of total

RNA was mixed with 1 μ l of 5 pM miRNA spike-in mix and dried in a table top speedvac (spike-in controls include: GCAAAGGCUAUCGUCAAGAGAUC; GUCGGCAUUUGGCUGGAACUUCAUA; UGACGGGUCUCUUCUUCGAUAGC; CAAAUCAACAAGAUGAGGUCUGGGG; CUUCCUGACCUUACCGAUUCCGA; UCAUUGCCUACAAGCCACCAAGC; GACAAAUCGGAUUCAAGGGCAGG; AGAUGUGGUUGCAACUUCGGAGC; UACCAACCCACCAAAACCAAACGU; UCCAAAACCAAACCAAUCCAAACC; ACAACCACUACUUCCGCCGUCAA; AACUCAAGCCGCCGGAUUCUUCA; AACACCCGUCAAGUCCAGUGCAU; UGCGCGGACUCCAACACUUUGUU, UGAUUGUUGUGACACCGGCACUACU). Each RNA pellet was fully resuspended in 25 μ l of hybridization buffer and denatured for 3 minutes at 95 °C. Until hybridization, the denatured samples were kept on ice. The success of the hybridization was monitored by cy3- and biotin-labeled hybridization controls including: [cy3]TCACTCATGGTTATGGCAGCACTGC,[bio]GTAGTTCGCCAGTTAATAGTTTGC G,[bio]TCTTACCGCTGTTGAGATCCAGTTC,[bio]CCCACCTCGTGCACCCAACTGATC TT,[bio]CCATCCAGTCTATTAATTGTTGCG. After 16 h hybridization at 42°C, the biochip was washed automatically and a program for signal enhancement was processed with the GRTA. Results were analyzed using the Geniom Wizard Software (febit biomed gmbh, Heidelberg). Then, for each array and each miRNA (feature), the median signal intensity of all pixels belonging to the respective feature was extracted from the raw data file such that for each miRNA five intensity values were calculated corresponding to each replicate copy of miRBase on the array.

Following background correction, median values were calculated from the five replicate intensity values of each miRNA in order to provide a stable estimate of the actual miRNA intensity value. To normalize arrays, Quantile Normalization was carried out using the freely available R software (<http://www.R-project.org>). All further analyses were carried out using

the normalized and background-subtracted intensity values. All microarray data were stored at the febit in-house database and can be accessed via the web-interface of the “Human Disease miRNome” project (<http://genetrail.bioinf.uni-sb.de/wholemirnومهproject>).

Statistical analysis

The approximate normal distribution of the measured data was verified by Shapiro-Wilk test followed by adjustment for multiple testing. Next, miRNAs showing a different behaviour in the two groups were identified by unpaired two-tailed parametric t-test. p-values obtained for each individual miRNA were adjusted for multiple testing by Benjamini-Hochberg (Benjamini et al. 2001) adjustment. Furthermore, the Area Under Curve (AUC) value of miRNAs was computed. AUC values are in the range from 0 to 1, where a value of 0.5 indicates equal distribution among healthy and diseased subjects which corresponds to the least diagnostic information of a biomarker. An AUC value of 1 means that all expression intensities of the respective miRNA are higher in glioblastoma samples (up-regulated miRNA) while an AUC of 0 means that all expression values of the miRNA are higher in controls (down-regulated biomarker).

In addition to the single biomarker analysis, samples were also classified according to miRNA patterns as calculated using Support Vector Machines (SVM) implemented in the R e1071 package (Häusler et al. 2010). For each classification, we determined the specificity, sensitivity and accuracy. Here, the specificity corresponds to the percentage of correctly identified controls, i.e., true negatives divided by true negative plus false positives. A true negative (TN) denotes a control that is actually recognized as control and a false positive (FP) represents a control predicted to be a glioblastoma patient. Similarly, the sensitivity corresponds to the percentage of correctly identified glioblastoma samples, i.e., true positives divided by true positives plus false negatives. A true positive (TP) denotes a glioblastoma

patient that is actually recognized as patient and a false negative (FN) represents a patient predicted to be healthy. The accuracy represents the fraction of correct predictions, i.e. $(TN + TP)/(TN + TP + FN + FP)$. Following classification, permutation tests were applied in order to check for putative overtraining. Here, the class labels were sampled at random and classifications were carried out using the permuted class labels. All statistical analyses were performed using version 2.11.1 of the R program (Keller et al. 2006).

Real-time RT-PCR

Real-time PCR was performed on the ABI PRISM 7000 Sequence Detection System using TaqMan microRNA assays for miR-128 and miR-342-3p (Applied Biosystems, Rotkreuz, Switzerland). RNU48 was assessed as endogenous control as previously described (Leidinger et al. 2010). The conditions for the PCR reactions were: 40 cycles, 95°C/15 s, 60°C/60 s. Data analysis was done using the $\Delta\Delta C_T$ method for relative quantification (Roth et al. 2010).

RESULTS

We obtained 20 blood samples from patients with a histopathologically confirmed diagnosis of glioblastoma. An overview of the patient characteristics is provided in Table 1. Healthy volunteers with a matched distribution of age and sex served as controls.

Altogether, 1158 mature miRNAs were quantitatively analyzed using a microarray platform. Following background subtraction and quantile normalization, all miRNAs with median intensity below 100 were considered lowly abundant and removed. The remaining 310 miRNAs were then analyzed using computational approaches. To capture the similarity of the samples, we computed all pair-wise correlation coefficients. For controls, we found on average a correlation of 0.901 and a standard deviation of 0.046, for glioblastoma patients a slightly decreased averaged correlation of 0.887 at a standard deviation of 0.047, showing the high biological (inter-individual) reproducibility of the measured miRNA profiles.

When comparing the filtered repertoire of the 310 miRNAs in glioblastoma patients and healthy controls, we observed a significant deregulation of 52 miRNAs, amounting to 16.8% of analyzed miRNAs. Of these, 27 miRNAs were up-regulated (52%) while 25 miRNAs were down-regulated (48%). The distribution of raw and respectively adjusted p-values across all miRNAs is presented as histogram plot in Figure 1. As shown there, after adjusting for multiple testing, a total of five miRNAs remained significant ($p < 0.05$). Especially two miRNAs were significant with a p-value of 0.025, namely miR-128, the most up-regulated and miR-342-3p, the most down-regulated miRNA, in the blood of tumor patients. miR-128 displayed a 1.7-fold increased median expression in samples from glioblastoma patients compared to healthy controls (intensity values of 665 vs. 397, $p = 0.0001$ by unadjusted and 0.025 by adjusted unpaired Student's t-test; AUC = 0.828). The expression level of miR-342-3p was decreased in the glioblastoma patients to 0.8 compared to the healthy donors (intensity values of 4738 vs. 5891, $p = 0.0002$ by unadjusted and 0.025 by adjusted unpaired Student's t-

test; AUC = 0.18). The expression intensity of both miRNAs is shown in Fig. 2A together with the respective receiver operating characteristic (ROC) curves (Fig. 2B). The deregulation of miR-128 and miR-342-3p was confirmed in a second cohort of glioblastoma patients and control donors by real-time PCR (Table 3A). Additionally, we assessed the expression of both markers separately by real-time PCR in the group of patients who had only undergone surgery at the time of blood sampling, as well as in the blood that had been obtained at least 6 months after surgery. Again, both miRNAs displayed a similar deregulation, independent of the time point of blood sampling or the recently received therapies (Table 3B).

The complete list of 52 miRNAs that are significantly deregulated in glioblastoma samples compared to controls, without adjusting for multiple testing, in the microarray analysis is shown in Table 4 (in order of increasing p-values). Although single deregulated miRNAs will hardly be useful as suitable biomarkers, they may provide interesting information related to the disease. A comparison of our data with databases linking miRNAs to specific diseases demonstrated that 4 of these miRNA had previously been associated with gliomas according to the Human miRNA and Disease Database (HMDD) (Lu et al. 2008; Jiang et al. 2009). These include hsa-miR-146b which inhibits glioma cell migration and invasion by targeting MMPs (Xia et al. 2009) and was up-regulated here. Moreover, hsa-miR-19a and 15a which have been shown to be up-regulated in glioblastoma cell lines (Chaudhry et al. 2010), are also up-regulated in our blood-based study. Finally, miR-128 (which is missing in HMDD) has been described as down-regulated in glioblastoma tissue compared to normal human brain (Ciafre et al. 2005), but was up-regulated in blood of glioblastoma patients here. Besides these glioblastoma-linked candidates, we also detected a multitude of miRNAs that have not been put into the context of glioblastoma or other diseases. Therefore, our large-scale approach revealed a panel of potentially interesting candidate miRNAs that may be involved in the malignant phenotype of glioblastomas.

In the next step, we aimed at identifying tumor-specific miRNA fingerprints that allow for a classification of the samples as glioblastoma or healthy controls. To this end, we applied statistical learning techniques such as SVM with different kernels and a filter based subset selection, as mentioned in the methods section. Classification was performed as previously described (Keller et al. 2006; Keller et al. 2009a). We carried out 20 iterations of a 10-fold cross validation and 20 non-parametric permutations with class labels that were randomly assigned to either the tumor or the control group. To determine a suitable subset of miRNA biomarkers, we started the training process with the 2 most significantly regulated markers and iteratively increased the number of miRNAs contributing to the classification (in order of increasing p-values). The performance of the classification for increasing subset sizes in terms of accuracy, specificity and sensitivity is provided in Figure 3. As detailed in this figure, a radial basis function SVM achieved the best result with a subset of 180 miRNAs. The classification of the blood samples as “glioblastoma” respective “control group” was done with an accuracy of 81% (95% CI 78%-84%), specificity of 79% (95% CI 75%-83%), and sensitivity of 83% (95% CI 71%-85%). This means that on average 3 glioblastoma patients were predicted to be controls (false negatives, FN), while 17 glioblastoma patients were correctly predicted as patients (true positives, TP). Likewise, on average 4 controls were predicted to be glioblastoma patients (false positives, FP), while 16 controls were correctly identified (true negatives, TN). Thus, in our study with just $n = 20$ glioblastoma and $n = 20$ control samples, the positive likelihood ratio, i.e., sensitivity divided by $(1 - \text{specificity})$ computes as 3.95 while the negative likelihood ratio defined as $(1 - \text{sensitivity})$ divided by specificity computes as 0.22.

One classification example for this statistical model is presented in Figure 4. Here, ‘C’s denote healthy donors while ‘D’ denote glioblastoma patients, the value on the y-axis corresponds to the logarithm of quotient of the probability being diseased and the probability

being healthy. All samples above the separating horizontal line are classified more likely to be patients, respectively all samples below the horizontal line to be controls.

In summary, the results of our analysis demonstrate a characteristic miRNA signature that can be obtained from the peripheral blood of glioblastoma patients.

DISCUSSION

Despite recent advances in the treatment of patients afflicted by glioblastoma, the prognosis remains poor (Stupp et al. 2009). Since glioblastoma is not a common disease, screening of asymptomatic individuals or patients with unspecific neurological symptoms and signs is not warranted. However, during the course of the disease, rapid tumor progression or recurrence are frequently observed. In addition to imaging studies that are normally only available every 2-3 months (Khayal et al. 2010), biomarkers might be helpful monitoring tools to detect tumor recurrence at the earliest time point or to distinguish between pseudoprogression and substantial tumor growth (Brandsma et al. 2008). The search for a blood-borne marker for glioblastoma has mostly focused on proteins (Hormigo et al. 2006; Gartner et al. 2010; Weller et al. 2010). However, *glial fibrillary acidic protein* (GFAP) which has been suggested as potential blood marker for gliomas (Jung et al. 2007) is rather unspecific since elevated GFAP levels are also observed in the blood of patients suffering from various different cerebral lesions (Foerch et al. 2006; Nylen et al. 2007). Likewise, other single proteins are lacking the required specificity and may also be difficult to detect due to posttranslational modifications. The determination of complex protein patterns in the blood, however, requires extensive technical procedures (Ludwig et al. 2008; Ludwig et al. 2009; Somasundaram et al. 2009). Consequently, the number of protein markers that are in clinical use is very limited and restricted to few diseases (Meany et al. 2009). In contrast, miRNAs have the advantage of being clearly defined markers that can easily be determined on the basis of microarrays or real-time PCR. The ease of multiplexing further opens the road for the analysis of specific miRNA patterns that comprise numerous miRNAs.

The present study aimed at laying a scientific basis for this approach and demonstrates that glioblastoma-specific miRNA fingerprints can be obtained from peripheral blood (Fig. 3 and 4). This approach is particularly promising since blood can easily be sampled and subjected to

analysis. The confirmation of the microarray data by real-time PCR (Table 3A) indicates that the miRNA analysis can simply be done by broadly available technologies that are already in clinical use.

Interestingly, we observed elevated expression levels of miR-128 in the blood of glioblastoma patients compared to healthy controls (Fig. 2). In contrast to our findings in the blood, miR-128 is down-regulated in glioblastoma tissue compared to normal human brain (Ciafre et al. 2005; Godlewski et al. 2008; Zhang et al. 2009). However, published signatures in blood, e.g. from multiple sclerosis and melanoma patients (Keller et al. 2009b; Leidinger et al. 2010), often show a non-significant overlap or even an inverse correlation with the tissue miRNA expression data that is available from the Human MiRNA Disease Database (HMDD) (Lu et al. 2008). For other diseases, tissue and blood show the same direction of deregulation, e.g. in non-small cell lung cancer (Shen et al. 2011) or arteriosclerosis obliterans (Li et al. 2011). Notably, the latter two studies did not consider, as in our case, blood cells but serum or plasma. Considering that *in silico* analysis using TargetScan predicts that several factors which are essential for TGF- β processing and signal transduction (furin, TGF- β receptor I, Smad2, Smad5, Smurf) are targeted by miR-128, while miR-128 expression in lymphocytes may be induced by TGF- β (data not shown), the functional impact of increased miR-128 levels in the blood of glioblastoma patients warrants further investigations. miR-342-3p, the second most deregulated miRNA (Fig. 2), has not yet been investigated in glioma tissues. An analysis in breast cancer tissue has, however, revealed a set of genes involved in cellular proliferation as potential targets of miR-342-3p (Van der Auwera et al. 2010). As with miR-128, the functional relevance of miR-342-3p down-regulation in blood cells requires further evaluation. Apart from these 2 miRNAs, we also noticed other putative differentially regulated miRNAs that have only partially been linked to pathological conditions. One reason for this might be the inclusion of recently described and still unexplored miRNAs in our array.

Considering the different treatment regimens that had been applied before inclusion in this study (Table 1 and 2), therapy-related effects can not be fully excluded. Recent findings suggest that changes in the miRNA repertoire of tumor cells may occur during chemotherapy (Rui et al. 2009; Zhou et al. 2010). However, while studies with larger patient cohorts and longitudinal blood sampling will be needed to fully address potential treatment-related effects on miRNAs in the blood of glioblastoma patients, these previous reports did not find miR-128 and miR-342-3p (the most significantly deregulated miRNA of our study) to be modulated by chemotherapy. Furthermore, miR-128 and miR-342-3p were deregulated in a similar way in blood that was obtained immediately after surgery versus blood from patients who had been operated more than 6 months previously and had received radio- and chemotherapy (Table 3B). This indicates that the detected differences are linked to the disease and not to a particular treatment. Suitably expanded studies might also allow to stratify miRNA patterns according to patient age and tumor size and thereby sharpen the respective profiles. However, even with the limitations imposed by this pilot study, we were able to reveal the presence of glioblastoma-associated miRNA fingerprints in the blood. The values computed for sensitivity, specificity and accuracy (Fig. 3) are comparable to those obtained by blood-derived miRNAs of ovarian or lung cancer patient cohorts (Keller et al. 2009a; Häusler et al. 2010). Further analyses demonstrated that miRNA fingerprints from the glioblastoma cohort can be distinguished from miRNA profiles described for lung and ovarian cancer blood samples (data not shown). Additionally, miRNA profiles from glioblastoma patients also differ from miRNA profiles of non-neoplastic diseases involving the nervous system such as multiple sclerosis (Keller et al. 2009b).

Due to the lack of any biomarker that is in clinical use for glioblastoma, the findings of our case-control study may lay the scientific basis for further studies using miRNAs for the follow-up of patients diagnosed with glioblastoma. Although the findings of our initial microarray screening were validated by real-time PCR, we are aware that our study still

represents a hypothesis generating proof-of-concept study on a rather small cohort and that these findings have to be validated on extended cohorts in order to advance the use of miRNAs as multiplex diagnostic tests for glioblastoma. Combination of this novel strategy with clinical and image-guided monitoring may help to improve the surveillance of glioblastoma patients during the course of the disease or the stratification of patients based on blood-derived profiles. Beyond a diagnostic multiplex test for glioblastoma, our research also opens new avenues for other ongoing research. Finally, the knowledge on altered miRNA profiles in immune cells might enable an improved understanding of glioblastoma-induced immunosuppression, which could on a very long term perspective lead to improved immunotherapeutic approaches.

In summary, blood-derived glioblastoma-associated miRNAs hold promise as biomarkers for the disease and warrant further exploration based on the findings of this pilot study.

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Conflict of interest

AK is affiliate of febit holding GmbH. All other authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Distribution of raw and adjusted p-values for the deregulation of individual miRNAs. 310 out of 1158 analysed miRNAs were abundant enough to allow for bioinformatic evaluation by Shapiro-Wilk test followed by an adjustment for multiple testing. Expression levels in the glioblastoma (n=20) and control (n=20) cohorts were compared by unpaired two-tailed parametric t-test and the number of individual p-values falling into the respective segments of width 0.05 were plotted as histogram (A). A similar histogram was generated from p-values obtained after Benjamini Hochberg adjustment for multiple comparisons (B). Vertical black lines denote the alpha level of 0.05.

Fig. 2. miR-128 and miR-342-3p are deregulated in glioma samples. The expression of miR-128 and miR-342-3p in blood samples of glioblastoma patients and healthy control donors was assessed by microarrays. A. Back-to-back histograms for miR-128 and miR-342-3p with glioblastoma samples (dark) and controls (pale). The y-axis shows the measured array intensity. B. ROC curves showing the true positive rate as function of the false positive rate are presented for miR-128 and miR-342-3p.

Fig. 3. Relationship between discriminatory power of miRNA profiles and size of the selected subsets. Support vector machines with different kernels were used to perform 20 iterations of a 10-fold cross validation, incorporating different numbers of miRNA. The plot shows the performance metrics of the classification using 22 miRNA subsets with increasing number of biomarkers. Accuracy (dark grey), specificity (light grey) and sensitivity (black) were evaluated.

Fig. 4. Classification of samples from glioblastoma patients or healthy controls. Based on the respective miRNA pattern, the quotient of the probability that the sample originates from a diseased patient divided by the probability that the donor is healthy is indicated for each sample. Samples with logarithms of above 0 (indicated by the dashed horizontal line) are more likely to be from diseased patients, i.e. to be glioblastoma samples. The vertical dashed line separates controls ('C') from glioblastoma samples ('D'). False negatives are marked by dotted circles (lower right corner) while false positives are marked by solid circles (upper left corner).

Tables:

Table 1: Patient characteristics

Number	age	sex	prior therapies
1	62	m	surgery
2	61	f	surgery
3	51	m	surgery, RT, TMZ
4	66	m	surgery, RT, TMZ
5	44	m	surgery
6	53	f	surgery, RT
7	48	m	surgery, RT, TMZ, BEV
8	54	f	surgery, RT, TMZ
9	51	m	surgery, RT
10	61	f	surgery, RT
11	54	f	surgery, RT, TMZ
12	63	m	surgery, RT, TMZ, BEV
13	53	f	surgery, RT, TMZ, BEV
14	46	m	surgery, RT, TMZ, gefitinib
15	52	m	surgery, RT, TMZ
16	51	m	surgery, RT, TMZ
17	60	f	surgery
18	59	m	surgery, RT, TMZ
19	55	m	surgery
20	59	f	surgery

RT: radiotherapy, TMZ: temozolomide, BEV: bevacizumab

Table 2: Patient characteristics

Number	age	sex	prior therapies
1	35	f	surgery, RT
2	52	m	surgery, RT, TMZ
3	66	m	surgery, RT, TMZ
4	37	m	surgery
5	59	m	surgery
6	74	m	surgery
7	60	m	surgery, RT
8	54	f	surgery, RT, TMZ
9	60	f	surgery, RT, TMZ
10	71	m	surgery

Table 3A: miRNA expression fold changes assessed by microarray and real-time RT-PCR

miRNA	fold change microarray	fold change real-time PCR
miR-128	1.68	1.50
miR-342-3p	0.80	0.76

Table 3B: miRNA expression fold changes assessed by real-time RT-PCR

miRNA	surgery only	> 6 months after surgery
miR-128	1.42	1.69
miR-342-3p	0.58	0.79

Table 4: miRNAs showing differential expression between glioblastoma samples and healthy control.

	median glioblastoma	median control	fold change	unadjusted p-value	adjusted p-value	AUC
hsa-miR-128	664.9	396.7	1.68	0.0001	0.0252	0.828
hsa-miR-342-3p	4738.1	5890.6	0.80	0.0002	0.0252	0.180
hsa-miR-194	7562.5	5173.6	1.46	0.0006	0.0407	0.789
hsa-life-6-3p	76.8	144.8	0.53	0.0006	0.0407	0.199
hsa-miR-628-3p	159.1	285.8	0.56	0.0007	0.0407	0.186
hsa-miR-148a	982.6	693.4	1.42	0.0017	0.0689	0.791
hsa-miR-30d	6044.9	3978.5	1.52	0.0019	0.0689	0.779
hsa-miR-574-3p	2508.0	5173.6	0.48	0.0020	0.0689	0.241
hsa-miR-223	2011.3	1026.5	1.96	0.0020	0.0689	0.804
hsa-miR-197	793.1	1178.3	0.67	0.0024	0.0734	0.225
hsa-miR-130b	1780.2	2850.1	0.62	0.0030	0.0801	0.221
hsa-miR-320b	3399.8	6206.3	0.55	0.0031	0.0801	0.240
hsa-miR-3195	144.3	211.1	0.68	0.0037	0.0877	0.246
hsa-miR-485-3p	81.9	130.0	0.63	0.0040	0.0879	0.233
hsa-miR-19a	2363.7	1717.0	1.38	0.0050	0.1032	0.746
hsa-miR-550*	637.8	365.8	1.74	0.0053	0.1032	0.748
hsa-miR-320a	20745.7	33712.7	0.62	0.0057	0.1047	0.251
hsa-miR-484	7407.5	4558.3	1.63	0.0061	0.1053	0.754
hsa-miR-941	89.1	133.3	0.67	0.0070	0.1079	0.228
hsa-miR-145	252.4	144.5	1.75	0.0072	0.1079	0.726
hsa-miR-151-3p	1057.0	617.0	1.71	0.0073	0.1079	0.794
hsa-miR-423-5p	6015.3	11139.6	0.54	0.0082	0.1134	0.251
hsa-miR-3196	435.1	810.5	0.54	0.0084	0.1134	0.274
hsa-miR-138-1*	81.8	135.7	0.60	0.0110	0.1395	0.215
hsa-miR-215	690.0	417.8	1.65	0.0114	0.1395	0.745
hsa-miR-548o	76.5	141.7	0.54	0.0117	0.1395	0.273
hsa-miR-362-3p	316.1	157.1	2.01	0.0147	0.1688	0.729
hsa-miR-3194	138.9	220.1	0.63	0.0155	0.1721	0.275
hsa-miR-486-3p	200.2	180.1	1.11	0.0167	0.1771	0.670
hsa-miR-15a	4378.5	3063.7	1.43	0.0171	0.1771	0.715
hsa-miR-30c	2243.8	1502.2	1.49	0.0191	0.1906	0.693
hsa-miR-4281	230.7	389.3	0.59	0.0204	0.1959	0.261
hsa-miR-1273d	90.9	127.3	0.71	0.0210	0.1959	0.263
hsa-miR-320c	1159.5	1690.9	0.69	0.0229	0.1959	0.293
hsa-miR-29c	393.8	228.1	1.73	0.0233	0.1959	0.686
hsa-miR-646	110.4	156.8	0.70	0.0234	0.1959	0.304
hsa-miR-424	248.4	185.6	1.34	0.0239	0.1959	0.711
hsa-miR-22	7252.6	6790.3	1.07	0.0240	0.1959	0.685
hsa-miR-4304	134.5	103.4	1.30	0.0252	0.2004	0.708
hsa-miR-30a	486.8	328.9	1.48	0.0287	0.2190	0.683
hsa-miR-320e	1211.5	1784.3	0.68	0.0291	0.2190	0.299
hsa-life-27	412.7	595.1	0.69	0.0297	0.2190	0.313
hsa-miR-149*	433.1	740.5	0.58	0.0317	0.2218	0.328
hsa-miR-331-3p	1222.4	939.1	1.30	0.0317	0.2218	0.671
hsa-miR-185	20745.7	18984.1	1.09	0.0322	0.2218	0.640
hsa-miR-1912	113.2	169.9	0.67	0.0343	0.2312	0.313
hsa-miR-1228*	908.1	1292.2	0.70	0.0365	0.2408	0.339

hsa-miR-146b-5p	183.2	156.0	1.17	0.0377	0.2435	0.635
hsa-miR-18a*	253.5	162.9	1.56	0.0405	0.2561	0.688
hsa-miR-1915	432.6	599.0	0.72	0.0419	0.2596	0.296
hsa-miR-100	164.4	334.3	0.49	0.0493	0.2951	0.358
hsa-miR-143	198.8	101.0	1.97	0.0497	0.2951	0.733

Annotation (Table 4):

The columns present median values for glioblastoma and control samples and the fold change (glioblastoma vs. control) represents their ratio. P-values were calculated using Student's t-test. Adjusted p-values were determined according to the method by Benjamini and Hochberg. AUC denote the area under the ROC curve. Significant miRNAs are highlighted in bold.